(54) Title: REPERFUSION THERAPY USING ANTIBODIES TO L-SELECTIN

(57) Abstract

The invention provides methods for prevention or treatment of reperfusion injury in a patient. In these methods, a therapeutically effective dose of an agent that specifically binds to L-selectin is administered. The agent functions by inhibiting adherence of leukocytes, particularly neutrophils, to endothelial cells. In preferred methods, the agent is a human or humanized antibody specific for L-selectin.

REPERFUSION THERAPY USING ANTIBODIES TO L-SELECTIN

5   This invention relates generally to therapeutic
methods using anti-L-selectin antibodies for preventing
ischemia/reperfusion induced tissue damage.

BACKGROUND OF THE INVENTION

10   Inflammation is a response of vascularized tissues
to infection or injury and is effected by adhesion of
leukocytes to the endothelial cells of blood vessels and their
infiltration into the surrounding tissues (Harlan, *Blood*
65:513-25 (1985)) (which is hereby incorporated by reference
15   in its entirety for all purposes). In normal inflammation,
the infiltrating leukocytes phagocytize invading organisms or
dead cells, and play a role in tissue repair and the immune
response. However, in pathologic inflammation, infiltrating
leukocytes release toxic mediators that can cause serious or
20   fatal damage. *See, e.g.,* Hickey, *Psychoneuroimmunology II*
(Academic Press 1990) (which is hereby incorporated by
reference in its entirety for all purposes).
           The attachment of leukocytes to endothelial cells is
effected via specific interaction of cell-surface ligands and
25   receptors, termed adhesion molecules, on endothelial cells and
leukocytes. *See, generally,* Springer, *Nature* 346:425-433
(1990). Adhesion molecules include integrins, selectins, and
immunoglobulin (Ig) superfamily members (*see* Springer, *supra,*
Osborn, *Cell* 62:3 (1990); Hynes, *Cell* 69:11 (1992), all of
30   which are incorporated herein by reference in their entirety
for all purposes).
           Integrins are heterodimeric transmembrane
glycoproteins consisting of an α chain (120-180 kDa) and a β
chain (90-110 kDa), generally having short cytoplasmic
35   domains. Th three known integrins, LFA-1, Mac-1 and P150,95,
have different alpha subunits, designat d CD11a, CD11b and
CD11c, and a common beta subunit designated CD18. LFA-1
($\alpha_L\beta_2$) is expressed on lymphocytes, granulocytes and

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monocytes, and binds predominantly to an Ig-family member
counter-receptor termed ICAM-1 (and perhaps to a lesser extent
ICAM-2). ICAM-1 is expressed on many cells, including
leukocytes and endothelial cells, and is up-regulated on
5   vascular endothelium by cytokines such as TNF and IL-1. Mac-1
($\alpha_M\beta_2$) is distributed on neutrophils and monocytes, and also
binds to ICAM-1 (and possibly ICAM-2). The third $\beta2$ integrin,
P150,95 ($\alpha_X\beta_2$), is also found on neutrophils and monocytes.
          The selectins, previously known as LECCAMs, consist
10   of L-selectin (also called LECAM-1, Mel-14 or LAM-1), E-
selectin (also called ELAM-1) and P-selectin (also called
GMP140 or PADGEM). E-selectin and P-selectin are both induced
on endothelial cells in response to cytokines. E-selectin is
preferentially expressed in cutaneous sites of inflammation
15   and may serve as an adhesion molecule for skin-homing T cells
in inflammatory response. (Picker et al., *Nature* 349:796
(1991), which is incorporated by reference in its entirety for
all purposes). E-selectin binds the carbohydrate group sialyl
Lewis x (sLex) (Lowe et al., *Cell* 63:475 (1990)), which is
20   incorporated by reference in its entirety for all purposes).
L-selectin is expressed on lymphocytes and neutrophils. The
cDNA sequence of human L-selectin has been published (EP
386,906). It has also been reported that L-selectin contains
a sialyl Lewis group.
25   A number of mouse antibodies to L-selectin have been
produced. *See* Kishimoto, *Proc. Natl. Acad. Sci. USA* 87:2244-
2248 (1990); Kishimoto et al., *Blood* 78:805-811 (1991); Tedder
et al., WO 93/02698, WO 93/00111 (each of which is
incorporated by reference in its entirety for all purposes).
30   Antibody binding studies have indicated that L-selectin
specifically interacts with, *inter alia*, E-selectin and P-
selectin (Picker et al., *Cell* 66:921 (1991), which is
incorporated by reference in its entirety for all purposes).
These analyses have also suggested possible roles for L-
35   selectin as (1) a T-cell homing receptor for the high
endoth lial venules of periph ral lymph nodes, and (2) a
neutrophil adhesion molecule having affinity for endothelial
cells (Hallmann et al., *Biochem. Biophys. Res. Commun.* 174:236

(1991)), which is incorporated by ref r nce in its ntirety for all purposes).

The identification of integrins and selectins as adhesive molecules effecting binding of neutrophils to endothelial cells suggests that these molecules might be potential therapeutic targets in treatment of inflammatory diseases. However, it is likely that the nature and degree of expression of these molecules varies for different cell subtypes and inflammatory stimuli. Thus, particular agents targeted against these various molecules are likely to be effective only for certain types of inflammatory conditions.

Ischemia/reperfusion injury is an inflammatory condition that occurs on restoring blood flow to organs suffering from an obstructed supply causing ischemia (oxygen deprivation). Unless rapidly relieved by reperfusion, ischemia causes death of surrounding cells, and eventually, death of a whole organ or patient. However, accumulating evidence suggests that reperfusion may itself exert deleterious effects on surrounding tissue. The deleterious effects of reperfusion are believed to result at least in part from an inflammatory response mediated by activated neutrophils in the restored blood flow.

Monoclonal antibodies that block the interaction of integrins with ICAM-1 have been reported to be somewhat successful in reducing myocardial tissue damage in animal models of the ischemia-reperfusion condition. These antibodies were specific either for CD18 (the common subunit of integrins) or against ICAM-1. Ma et al, *J. Clin. Invest.* 88:1237-1243 (1991); Ma et al, *Circulation* 86:937-46 (1992) (each of which is incorporated by reference in its entirety for all purposes). However, it is possible that other adhesion molecules besides the integrins and their receptors play a role in ischemia-reperfusion injury. Agents targeted against such adhesion molecules would considerably augment th repertoire of possibl therapeutic treatments. Combinations of therapeutic agents may be more effective than either agent alone. Moreover, the availability of alternative agents

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provides treatment options should any one agent induce
intol rance in a particular pati nt.

      Based on the foregoing, it is apparent that a need
exists for additional and more effective therapeutic agents
5   and methods for treatment of ischemia-reperfusion injury. The
present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

      The invention provides methods for prevention or
10 treatment of reperfusion injury in a patient. In these
methods, a therapeutically effective dose of an agent that
specifically binds to L-selectin is administered. The agent
may be administered directly to the patient or to an isolated
organ awaiting transplant into the patient. The agent
15 functions by inhibiting adherence of leukocytes, particularly
neutrophils, to endothelial cells. The agents include
antibodies that specifically bind to L-selectin. Usually, the
methods are performed using monoclonal antibodies. Humanized
or human antibodies are preferred. Some antibodies compete
20 with an antibody designated hu DREG-200 for specific binding
to L-selectin. The methods are particularly useful for
treating patients suffering from myocardial ischemia. In some
methods, administration of an agent specific for L-selectin is
combined with administration of a therapeutically effective
25 dose of a second agent. For example, in some methods, the
second agent is a thrombolytic agent and in other methods, the
agent is an antibody specific for CD18.

BRIEF DESCRIPTION OF THE DRAWINGS

30   Figure 1: Amino acid sequences of the mature light
chain (A) and heavy chain (B) variable regions of the mouse
DREG-200 antibody (upper lines) and humanized DREG-200
antibody (hu DREG-200) (lower lines). The three CDRs in each
chain are underlined. Residues in the framework that have
35 been replaced with mouse amino acids or typical human amino
acids in the humanized antibody are double underlined.

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Figur 2: Inhibition of *in vitro* binding of
neutrophils to ischemia-reperfusion-activated endothelial
cells by monoclonal antibody hu DREG-200. LCX, left
circumflex coronary artery; LAD, left anterior descending
5   coronary artery.

Figure 3: *In vivo* protection of myocardial tissue
from ischemia-reperfusion injury by monoclonal antibody hu
DREG-200. Tissue damage was assayed by staining with
nitroblue tetrazolium.

10   Figure 4: *In vivo* protection of myocardial tissue
from ischemia-reperfusion injury by monoclonal antibody hu
DREG-200. Tissue damages was assayed by determining plasma
creatine kinase activity (CK). O = occlusion, R =
reperfusion.

15   Figure 5: Inhibition of neutrophil accumulation at
ischemic endothelial cells, by hu DREG-200, during
reperfusion. Neutrophils were quantified from MPO activity.

Figure 6: Improved mechanical performance of the
heart during reperfusion of ischemic myocardial tissue
20   conferred by hu DREG-200 treatment. Data are expressed as
percentages of initial values.

Figure 7: Reduction of coronary endothelial cell
dysfunction in ischemic-reperfused tissue by treatment with hu
DREG-200.

25   DEFINITIONS
The term "substantial identity" or "substantial
homology" means that two peptide sequences, when optimally
aligned, such as by the programs GAP or BESTFIT using default
gap weights, share at least 80 percent sequence identity,
30   preferably at least 90 percent sequence identity, more
preferably at least 95 percent sequence identity or more
(*e.g.*, 99 percent sequence identity). Preferably, residue
positions which are not identical differ by conservative amino
acid substitutions.

35   For purposes of classifying amino acids
substitutions as conservative or nonconservative, amin acids
are group d as follows: Group I (hydrophobic sidechains):
norleucine, met, ala, val, leu, ile; Group II (neutral

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hydrophilic side chains): cys, ser, thr; Group III (acidic
side chains): asp, glu; Group IV (basic side chains): asn,
gln, his, lys, arg; Group V (residues influencing chain
orientation): gly, pro; and Group VI (aromatic side chains):

5   trp, tyr, phe. Conservative substitutions involve
substitutions between amino acids in the same class. Non-
conservative substitutions constitute exchanging a member of
one of these classes for another.

        Amino acids from the variable regions of the mature

10   heavy and light chains of immunoglobulins are designated Hx
and Lx respectively, where x is a number designating the
position of an amino acid according to the scheme of Kabat,
Sequences of Proteins of Immunological Interest (National
Institutes of Health, Bethesda, MD (1987) and (1991)

15   (incorporated by reference in their entirety for all
purposes). Kabat lists many amino acid sequences for
antibodies for each subclass, and lists the most commonly
occurring amino acid for each residue position in that
subclass. Kabat uses a method for assigning a residue number

20   to each amino acid in a listed sequence, and this method for
assigning residue numbers has become standard in the field.
Kabat's scheme is extendible to other antibodies not included
in his compendium by aligning the antibody in question with
one of the consensus sequences in Kabat. The use of the Kabat

25   numbering system readily identifies amino acids at equivalent
positions in different antibodies. For example, an amino acid
at the L50 position of a human antibody occupies the
equivalent position to an amino acid position L50 of a mouse
antibody.

30   From N-terminal to C-terminal, both light and heavy
chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3
and FR4. The assignment of amino acids to each domain is in
accordance with the structural definition of Kabat (1987 and
1991), *supra*, or Chothia & Lesk, *J. Mol. Biol.* 196:901-917

35   (1987); Chothia et al. *Nature* 342:878-883 (1989) (incorporated
by reference in their entirety for all purposes).

DETAILED DESCRIPTION

The invention provid s methods of reperfusion therapy and pharmaceutical compositions for use in the same. The pharmaceutical compositions contain agents that specifically bind to L-selectin. The agents function by preventing leukocytes, particularly neutrophils, bearing L-selectin on their surface from interacting with endothelial cells, and thereby initiating an inflammatory response.

I. Active Agents

The invention provides antibodies specific for L-selectin, and other molecules, such as miniproteins, that have a similar binding specificity, for use in therapeutic methods and pharmaceutical compositions.

A. Antibodies

1. General Characteristics

The basic antibody (or immunoglobulin) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The NH_2-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH part of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (See, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pp. 131-166, Raven Press, N.Y. (1984), which is inc rp rated by reference in its entir ty for all purposes.)

The variable regions of each light/heavy chain pair form the antibody binding sit . The chains all exhibit the

same general structure of relatively conserved framework
regions joined by three hypervariable regions, also called
Complementarity Determining Regions or CDRs (see "Sequences of
Proteins of Immunological Interest," Kabat, E., et al., U.S.

5   Department of Health and Human Services (1987); and Chothia
and Lesk, J. Mol. Biol., 196:901-917 (1987), which are
incorporated by reference in their entirety for all purposes).
The CDRs from the two chains of each pair are aligned by the
framework regions, enabling binding to a specific epitope.

10   The term "antibody" or "immunoglobulin" is used
generically to refers to a protein consisting of one or more
polypeptides substantially encoded by immunoglobulin genes.
The recognized immunoglobulin genes include the kappa, lambda,
alpha, gamma, delta, epsilon and mu constant region genes, as

15   well as the myriad immunoglobulin variable region genes.
Thus, the term "antibody" includes not only intact antibodies,
but, for each intact antibody discussed, a variety of
fragments having the same or similar binding specificity as
the intact antibody. Fragments include Fv, Fab, and (Fab')$_2$

20   as well as bifunctional hybrid antibodies (e.g., Lanzavecchia
et al., Eur. J. Immunol. 17:105 (1987)) and single chains
(e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85:5879-
5883 (1988) and Bird et al., Science, 242:423-426 (1988),
which are incorporated by reference in their entirety for all

25   purposes). (See, generally, Hood et al., Immunology,
Benjamin, N.Y., 2nd ed. (1984), Harlow and Lane, Antibodies.
A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and
Hunkapiller and Hood, Nature, 323:15-16 (1986), which are
incorporated by reference in their entirety for all purposes).

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2.   Binding Characteristics

                The agent of the invention should bind to an epitope
of L-selectin so as to inhibit adhesion of neutrophils to
endothelial cells. Many antibodies are targeted against L-

35   selectin epitope(s) that are responsible for (i.e.,
participate directly in) binding of leukocytes to endothelial
cells. However, other agents are targeted against neighboring
epitopes and function by steric hindrance of th epitope(s)

that directly participate in binding. The, term "L-selectin," encompasses a human L-selectin polypeptide having the amino acid sequence presented in Tedder, EPA 386,906 (1990), allelic and nonallelic variants thereof, cognate variants, such as the mouse MEL-14 receptor, and natural and induced mutants of any of these. L-selectin is a type I membrane receptor having a molecular weight of about 80-85 kDa. The intact polypeptide has been subdivided into the following domains: a lectin domain, an EGF-like domain, a first short consensus domain, a second consensus repeat domain, a leader sequence domain, a transmembrane domain and a phosphorylation domain. See Tedder et al, WO 93/02698.

One example of an antibody having the required binding specificity is the antibody DREG-200. See Kishimoto et al., *Proc. Natl. Acad. Sci.*, *supra* (mouse antibody) and Co et al., copending application, USSN 07/983,946 (humanized antibody, hereinafter sometimes "hu DREG-200") (which are hereby incorporated by reference in their entirety for all purposes). Other antibodies having the same or similar epitope specificity can be isolated by competitive binding experiments with DREG-200 (mouse or humanized). In competitive binding experiments, the L-selectin molecule can be expressed on the surface of neutrophils or from transformed COS cells. Alternatively, purified recombinant L-selectin, or a binding domain thereof, can be immobilized to a solid support.

Antibodies having the required epitope specificity can also be identified by direct screening for the capacity to block neutrophil-endothelial cell interaction. A simple visual assay for detecting such interaction has been described by Kishimoto (1991), *supra*. Briefly, monolayers of human umbilical vein cells are stimulated with IL-1. Neutrophils, with or without pretreatment with the antibody under test, are added to the monolayer under d fin d conditions, and the numb r of adh ring neutrophils is determined microscopically. In one method, the neutrophils are obtained from human leukocyte adhesion deficient patients. See Anderson et al., *Ann. Rev. Med.* 38:175 (1987). The neutrophils from such

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patients lack integrin receptors, whose binding to neutrophils
might obscure the effects of blocking L-selectin binding.

An alternative and preferred method of screening for
suitable antibodies is described in Example 2. In this
5   method, endothelial cells for use in the adhesion assay are
activated *in vivo* by ischemia-reperfusion of laboratory
animals. Tissue bearing endothelial cells is extracted and
incubated with labelled neutrophils, with and without
pretreatment with test antibody, *in vitro*. Neutrophils
10   attaching to endothelial cells are detected by fluorescence
microscopy.

An alternative approach for identifying antibodies
having the desired specificity is to map the L-selectin amino
acid residues required for binding to an exemplary antibody,
15   such as DREG-200 (mouse or humanized), known to have
appropriate specificity. The epitope bound by DREG-200 or any
other anti-L-selectin antibody is determined by providing a
family of fragments containing different amino acid segments
from L-selectin. Each fragment typically comprises at least
20   4, 6, 8, 10, 20, 50 or 100 contiguous amino acids.
Collectively, the family of polypeptide covers much or all of
the amino acid sequence of a full-length L-selectin peptide.
Members of the family are tested individually for binding to,
e.*g*., the DREG-200 antibody (mouse or humanized) by, e.*g*.,
25   immunoprecipitation or Western blotting. The smallest
fragment that can specifically bind to the antibody under test
delineates the amino acid sequence of the epitope recognized
by the antibody. Alternatively, epitopes can be identified by
protein footprinting of L-selectin polypeptides. In this
30   technique, an L-selectin polypeptide is allowed to bind to an
antibody and then exposed to a protease. The residues of L-
selectin binding to the monoclonal antibody are protected from
proteolytic degradation, and identified by amino acid
sequencing. L-selectin fragments consisting essentially of
35   the epitope(s) identified by the above analyses, ar suitable
immunogens to be used in the methods for producing antibodies
discussed *infra*.

The strength of antibody binding to its antigen
provides an indication of therapeutic potential, with stronger
binding likely to be more useful. Usually, the antibodies
will have an affinity of at least about 10^6 M^{-1}, more usually

5   at least about 10^8 M^{-1} and preferably at least about 10^9 M^{-1}
for L-selectin. For example, mouse DREG-200 binds with an
affinity of about 10^8 M^{-1}. Other preferred antibodies will
have binding affinities within a factor of about three of that
of mouse or hu DREG-200, or higher.

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3.   Production of Antibodies
a.   Nonhuman Antibodies
The production of non-human monoclonal antibodies,
e.g., murine, rat and so forth, is well known and may be

15   accomplished by, for example, immunizing the animal with a
preparation containing an L-selectin polypeptide or an
immunogenic fragment thereof. Particularly, useful as
immunogens are cells naturally expressing or stably
transfected with recombinant L-selectin and expressing L-

20   selectin receptors on their cell surface. Antibody-producing
cells obtained from the immunized animals are immortalized and
screened for the production of an antibody which binds to L-
selectin. *See* Harlow & Lane, *Antibodies, A Laboratory Manual*
(C.S.H.P. NY, 1988) (incorporated by reference in its entirety

25   for all purposes).
A number of murine antibodies to L-selectin have
already been produced. These include mouse DREG-55, -56,
-110, -152 and 200 discussed by Kishimoto et al, *Proc. Natl.*
Acad Sci. USA 87:2244-2248 (1990) and anti-LAM-1, -2, -4, -5,

30   -6, -8, -10, 11, -14 and -15 discussed by Tedder et al, WO
93/02698. The DREG-55 and -56 antibodies are likely to be
effective in the present methods because they have been shown
to block binding of neutrophils to endothelial cells *in vitro*.
Nonhuman antibodies produced by the above methods

35   are generally not pr f rred for use in therapeutic m th ds.
However, such antibodies are useful for generating humaniz d
and, in s m instances, human antibodi s by the pr cedures
discussed *infra*.

b.   Humanized and Human Antibodies

These antibodies have at least three potential
advantages over nonhuman antibodies, such as mouse antibodies,
5   for use in human therapy:

1)   because the effector portion is human, it may
interact better with the other parts of the
human immune system (e.g., destroy the target
cells more efficiently by complement-dependent
10   cytotoxicity (CDC) or antibody-dependent
cellular cytotoxicity (ADCC));

2)   the immune response against human or humanized
antibodies should be minimal or absent;

3)   human and humanized antibodies are expected to
15   have a longer half life than mouse antibodies
in the human circulation (see Shaw et al, J.
Immunol. 138:4534-4538 (1987)).

(1)   Humanized Antibodies

20   Humanized immunoglobulins are produced by linking
the CDR regions of non-human antibodies known to have useful
properties to human constant regions by recombinant DNA
techniques. See Queen et al., Proc. Natl. Acad. Sci. USA
86:10029-10033 (1989) and WO 90/07861 (incorporated by
25   reference in their entirety for all purposes). The humanized
immunoglobulins have variable region framework residues
substantially from a human immunoglobulin (termed an acceptor
immunoglobulin) and complementarity determining regions
substantially from a nonhuman immunoglobulin, e.g., the mouse
30   DREG-200 antibody (referred to as the donor immunoglobulin).
The constant region(s), if present, are also substantially
from a human immunoglobulin.

The human variable domains are usually chosen from
human antibodies whose framework sequences exhibit a high
35   degree of sequenc id ntity with the murine variable regi n
domains from which the CDRs were derived. The heavy and light
chain variable framework r gions can be derived from the same
or different human antibody sequences. Indeed, the heavy and

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light chain framework regions can each be deriv d from m re
than one human antibody. The human antibody sequences can b
the sequences of naturally occurring human antibodies or can
be consensus sequences of several human antibodies. *See*
5   Carter et al., WO 92/22653 (1992) (incorporated by reference
in its entirety for all purposes).

The unnatural juxtaposition of murine CDR regions
with human variable framework region can result in unnatural
conformational restraints, which, unless corrected by
10   substitution of certain amino acid residues, lead to loss of
binding affinity. The selection of amino acid residues for
substitution is determined, in part, by computer modelling.
Computer hardware and software for producing three-dimensional
images of immunoglobulin molecules are widely available. In
15   general, molecular models are produced starting from solved
structures for immunoglobulin chains or domains thereof. The
chains to be modelled are compared for amino acid sequence
similarity with chains or domains of solved three dimensional
structures, and the chains or domains showing the greatest
20   sequence similarity is/are selected as starting points for
construction of the molecular model. The solved starting
structures are modified to allow for difference between the
actual amino acids in the immunoglobulin chains or domains
being modelled, and those in the starting structure. The
25   modified structures are then assembled into a composite
immunoglobulin. Finally, the model is refined by energy
minimization and by verifying that all atoms are within
appropriate distances from one another and that bond lengths
and angles are within chemically acceptable limits.
30   Additional models can be constructed representing the
structure when further amino acid substitutions to be
discussed *infra*, are introduced.

In general, substitution of human amino acid
residues with murine should be minimized, because introducti n
35   of murine residues increases the risk of the antibody
eliciting a HAMA r sponse in humans. Amino acids are selected
for substitution bas d on their possible influence on CDR
conformation and/or binding to antigen. Inv stigation of such

possible influences is by modelling, examination of the
characteristics of the amino acids at particular locations, or
empirical observation of the effects of substitution or
mutagenesis of particular amino acids.

5   When an amino acid differs between a mouse variable
framework region and an equivalent human variable framework
region, the human framework amino acid should usually be
substituted by the equivalent mouse amino acid if it is
reasonably expected that the amino acid:

10   (1)   noncovalently binds antigen directly;
                 (2)   is adjacent to a CDR region or otherwise
interacts with a CDR region (e.g., is within about 4-6 Å of a
CDR region).

            Other candidates for substitution are acceptor human
15   framework amino acids that are unusual for a human
immunoglobulin at that position. These amino acids can be
substituted with amino acids from the equivalent position of
more typical human immunoglobulins. Alternatively, amino
acids from equivalent positions in the mouse donor antibody
20   can be introduced into the human framework regions when such
amino acids are typical of human immunoglobulins at the
equivalent positions.

            In general, substitution of all or most of the amino
acids fulfilling the above criteria is desirable.
25   Occasionally, however, there is some ambiguity about whether a
particular amino acid meets the above criteria, and
alternative variant immunoglobulins are produced, one of which
has that particular substitution, the other of which does not.

            Usually the CDR regions in humanized antibodies are
30   substantially identical, and more usually, identical to the
corresponding CDR regions in the mouse donor antibody (e.g.,
mouse DREG-200). Occasionally, however, it is desirable to
change one of the residues in a CDR region, for example, to
create a resemblance to the binding site of a ligand of L-
35   sel ctin. Although not usually desirable, it is sometimes
possible to make one or more conservative amino acid
substitutions of CDR residues without appreciably affecting
th binding affinity of the resulting humanized immunoglobulin.

Other than for th specific amino acid substitutions discussed above, the framework r gions of humaniz d immunoglobulins are usually substantially identical, and more usually, identical to the framework regions of the human antibodies from which they were derived. However, in some embodiments the framework regions can vary from the native sequences at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Of course, many of the amino acids in the framework region make little or no direct contribution to the specificity or affinity of an antibody. Thus, many individual conservative substitutions of framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized immunoglobulin. However, in general, such substitutions are undesirable. Modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see Gillman & Smith, *Gene* 8:81-97 (1979) and Roberts et al., *Nature* 328:731-734 (1987), both of which are incorporated by reference in their entirety for all purposes).

The amino acids sequences of the variable regions of an exemplary humanized antibody, designated hu DREG-200, are shown in Fig. 1. This antibody was constructed by fusing the CDR regions of the murine DREG-200 antibody with the framework regions of the human Eu antibody. Human light chain residues at positions L87, L54, L66, L76 and L93, were replaced with corresponding murine residues. Human heavy chain residues at positions H93, H95, H98, H111, H112, H115, H30, H98, H111, H27, H48, and H72 were replaced with corresponding murin residues. Human heavy chain residue H116 was replac d with an amino acid more typical of human immunoglobulin heavy chains at that position.

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The variable segments of humanized antibodies
produced as described *supra*, are typically linked to at least
a portion of an immunoglobulin constant region (Fc), typically
that of a human immunoglobulin. Human constant region DNA

5   sequences can be isolated in accordance with well-known
procedures from a variety of human cells, but preferably
immortalized B-cells (*see* Kabat, *supra*, and WO87/02671).
Ordinarily, the antibody will contain both light chain and
heavy chain constant regions. The heavy chain constant region

10   usually includes CH1, hinge, CH2, and CH3 regions.

The humanized antibodies include antibodies having
all types of constant regions, including IgM, IgG, IgD, IgA
and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4.
When cytotoxic activity is not desirable, the constant domain

15   may be of the IgG4 class.

(2)   Human Antibodies

Human antibodies can now be produced by a variety of
techniques. (*See, e.g.,* Larrick et al., U.S. Patent No.

20   5,001,065, for review, which is hereby incorporated by
reference in its entirety for all purposes). Preferred
techniques for generation of human anti-L-selectin antibodies
are discussed below.

25   (a)   *Trioma Methodology*. The basic approach and an
exemplary cell fusion partner, SPAZ-4, for use in this
approach have been described by Oestberg et al., *Hybridoma*
2:361-367 (1983); Oestberg, U.S. Patent No. 4,634,664; and
Engleman et al., US Patent 4,634,666 (each of which is

30   incorporated by reference in its entirety for all purposes).
The antibody-producing cell lines obtained by this method are
called triomas, because they are descended from three cells--
two human and one mouse. Initially, a mouse myeloma line is
fused with a human B-lymphocyte to obtain a non-antibody-

35   producing xenogeneic hybrid cell, such as the SPAZ-4 cell line
described by Oestberg, *supra*. The xenogeneic cell is th n
fused with an immunized human B-lymphocyte to obtain an
antibody-producing trioma cell line. Triomas have been found

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to produce antibody more stably than ordinary hybridomas mad from human cells.

The immunized B-lymphocytes are obtained from the blood, spleen, lymph nodes or bone marrow of a human donor. *In vivo* immunization of a living human with L-selectin is usually undesirable because of the risk of initiating a harmful response. Thus, B-lymphocytes are usually immunized *in vitro* with an L-selectin polypeptide, an antigenic fragment thereof or a cell bearing either of these. If antibodies against a specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof for *in vitro* immunization. B-lymphocytes are typically exposed to antigen for a period of 7-14 days in a media such as RPMI-1640 (*see* Engleman, *supra*) supplemented with 10% human plasma.

The immunized B-lymphocytes are fused to a xenogeneic hybrid cell such as SPAZ-4 by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37 degrees, for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids (*e.g.*, HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to L-selectin or a fragment thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown *in vitro* in culture medium. The trioma cell lines obtained are then tested for the ability to bind L-selectin or a polypeptide thereof.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into a suitable cell line for expression of recombinant immunoglobulins. *See, e.g.*, Co et al., *J. Immunol.*, 148:1149 (1992) (incorporated by reference in its entirety for all purposes).

(b) *Transg nic Non-Human Mammals*. Human antibodies against L-selectin can also be produced from non-human

transgenic mammals having transgenes encoding at least a
s gm nt of th human immunoglobulin locus. Usually, the
endogenous immunoglobulin locus of such transgenic mammals is
functionally inactivated. Preferably, the segment of the
5   human immunoglobulin locus includes unrearranged sequences of
heavy and light chain components. Inactivation of endogenous
immunoglobulin genes and introduction of exogenous
immunoglobulin genes can be achieved by targeted homologous
recombination, or by the introduction of YAC chromosomes. The
10   transgenic mammals resulting from this process are capable of
functionally rearranging the immunoglobulin component
sequences, and expressing a repertoire of antibodies of
various isotypes encoded by human immunoglobulin genes,
without expressing endogenous immunoglobulin genes. The
15   production and properties of mammals having these properties
are described in detail by, e.g., Lonberg et al., WO93/12227
(1993); Kucherlapati, WO 91/10741 (1991) (each of which is
incorporated by reference in its entirety for all purposes).
Transgenic mice are particularly suitable. Anti-L-selectin
20   antibodies are obtained by immunizing a transgenic nonhuman
mammal, such as described by Lonberg or Kucherlapati, supra,
with L-selectin or a fragment thereof. Monoclonal antibodies
are prepared by, e.g., fusing B-cells from such mammals to
suitable myeloma cell lines using conventional Kohler-Milstein
25   technology.
          c.   Phage Display Methods: A further approach for
obtaining human anti-L-selectin antibodies is to screen a DNA
library from human B cells according to the general protocol
outlined by Huse et al., Science 246:1275-1281 (1989).
30   Antibodies binding to L-selectin or a fragment thereof are
selected. Sequences encoding such antibodies (or a binding
fragments) are then cloned and amplified. The protocol
described by Huse is rendered more efficient in combination
with phage-display technology. See, e.g., Dower et al., WO
35   91/17271 and McCafferty et al., WO 92/01047 (each of which is
incorporated by reference in its entir ty for all purposes).
In these methods, libraries of phage are produced in which
members display different antibodies on their outersurfaces.

Antibodies are usually displayed as Fv or Fab fragments.
Phage displaying antibodies with a desired specificity are
selected by affinity enrichment to an L-selectin polypeptide
or fragment thereof.

5   In a variation of the phage-display method, human
antibodies having the binding specificity of a selected murine
antibody can be produced. See Winter, WO 92/20791. In this
method, either the heavy or light chain variable region of the
selected murine antibody (e.g., mouse DREG-200) is used as a
10   starting material. If, for example, a light chain variable
region is selected as the starting material, a phage library
is constructed in which members displays the same light chain
variable region (i.e., the murine starting material) and a
different heavy chain variable region. The heavy chain
15   variable regions are obtained from a library of rearranged
human heavy chain variable regions. A phage showing strong
specific binding for L-selectin (e.g., at least about 10^8 and
preferably at least about 10^9 M^{-1}) is selected. The human
heavy chain variable region from this phage then serves as a
20   starting material for constructing a further phage library.
In this library, each phage displays the same heavy chain
variable region (i.e., the region identified from the first
display library) and a different light chain variable region.
The light chain variable regions are obtained from a library
25   of rearranged human variable light chain regions. Again,
phage showing strong specific binding for L-selectin are
selected. These phage display the variable regions of
completely human anti-L-selectin antibodies. These antibodies
usually have the same or similar epitope specificity as the
30   murine starting material (e.g., mouse DREG-200).

        B.   Other Therapeutic Agents
             Having produced an antibody having desirable
properties, such as DREG-200 (mouse or humanized), oth r
35   nonantib dy agents having similar binding sp cificity/and or
affinity can be produced by a variety of methods. For
example, Fodor et al., US 5,143,854, discuss a technique
termed VLSIPS™, in which a divers collection of short

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p ptides are formed at selected positions on a solid
substrate. Such peptides could then be screened for binding
to the L-selectin epitope recognized by DREG-200, optionally
in competition with the DREG-200 antibody (mouse or
5   humanized). Libraries of short peptides can also be produced
and screened using phage-display technology. *See, e.g.,*
Devlin WO91/18980. Fragments of a ligand of L-selectin that
compete with the ligand for binding to neutrophils bearing L-
selectin are also suitable agents for use in the present
10   method. Optionally, these fragments, or indeed polypeptides
produced by other methods, can be variegated to achieve
improved binding affinity for L-selectin. *See, e.g.,* Ladner,
US 5,223,409 (which is hereby incorporated by reference in its
entirety for all purposes).

15

II. Pharmaceutical Compositions

        The pharmaceutical compositions for use in the
therapeutic methods discussed *infra,* typically comprises an
active agent, such as an anti-L-selectin antibody, dissolved
20   in an acceptable carrier, preferably an aqueous carrier. Some
compositions contain a cocktail of multiple active agents, for
example, an anti-L-selectin antibody and a thrombolytic agent.
A variety of aqueous carriers can be used, *e.g.,* water,
buffered water, phosphate buffered saline (PBS), 0.4% saline,
25   0.3% glycine, human albumin solution and the like. These
solutions are sterile and generally free of particulate
matter. The compositions may contain pharmaceutically
acceptable auxiliary substances as required to approximate
physiological conditions such as pH adjusting and buffering
30   agents, toxicity adjusting agents and the like, for example,
sodium acetate, sodium chloride, potassium chloride, calcium
chloride and sodium lactate. The concentration of antibody in
these formulations can vary widely, *i.e.,* from less than about
0.005%, usually at least about 1% to as much as 15 or 20% by
35   weight and will be selected primarily based on fluid volumes,
viscosities, and so forth, in accordance with the particular
mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-10 mg of immunoglobulin. A typical composition for intravenous infusion could be made up to contain 250 ml of

5   sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science* (15th ed., Mack Publishing Company,

10 Easton, PA, 1980), which is incorporated by reference in its entirety for all purposes. Compositions suitable for lavage or other routes will be selected according to the particular use intended.

Therapeutic agents of the invention can be frozen or

15 lyophilized for storage and reconstituted in a suitable carrier prior to use. Lyophilization and reconstitution can lead to varying degrees of antibody activity loss (*e.g.*, with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies). Dosages may have

20 to be adjusted to compensate.

III.   Therapeutic Methods

The invention provides methods for reperfusion therapy using the therapeutic agents and pharmaceutical

25 conditions discussed *supra*. The methods are particularly useful for therapeutic and prophylactic treatment of ischemia-reperfusion injury caused by, for example, myocardial infarction, cardiac surgery, such as coronary artery bypass or elective angioplasty, cerebral ischemic event (*e.g.*, a stroke

30 or brain surgery), renal, splenic or hepatic ischemic events, shock (*e.g.*, hemorrhagic-shock-induced gastric mucosal injury), and the like.

The methods are particularly useful for humans, but may also be practiced on veterinary subjects. The therapeutic

35 methods ar usually applied to organs pr sent in living subjects. However, the methods are equally applicable to dissected organs, particularly when such organs are awaiting transplant to a recipient patient.

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The therapeutic agents and compositions targeted
against L-selectin can also be us d in combination with agents
targeted against other molecules, particularly humanized or
human antibodies reactive with different adhesion molecules.
5   Suitable immunoglobulins include those specific for CD11a,
CD11b, CD18, E-selectin, P-selectin and ICAM-1. The
immunoglobulins should bind to epitopes of these adhesion
molecules so as to inhibit binding of leukocytes, particularly
neutrophils, to endothelial cells. The combination of
10   therapeutic agents against L-selectin with agents against CD18
is particularly preferred. It has been reported that L-
selectin and CD18 mediate sequential stages in the neutrophil-
endothelial cell attachment process. *See* von Andrian et al.,
Proc. Natl. Acad. Sci. USA 88:7538 (1991); Luscinskas, *J.*
15   *Immunol.* 142:2257 (1989) (each of which is incorporated herein
by reference in its entirety for all purposes). Anti-CD18
antibodies are described in, *e.g.*, EPA 440,351 and US
4,797,277 (incorporated by reference in their entirety for all
purposes). Thus, a combination of agents directed against
20   both targets is likely to provide a particularly effective
reperfusion therapy. Other suitable antibodies for use in
combination therapies are those specific for lymphokines, such
as IL-1, IL-2 and IFN-γ, and their receptors. Such antibodies
serve to block activation of endothelial cells, and thereby
25   prevent their interaction with neutrophils in an inflammatory
response.

In some methods of reperfusion therapy, therapeutic
agents directed against L-selectin are used in combination
with thrombolytic agents. The thrombolytic agent serves to
30   remove a blockage to the blood supply, while the anti-L-
selectin agent protects against tissue injury resulting from
restoration of the blood supply. Thrombolytic agents are
drugs having the capacity, directly or indirectly, to
stimulate dissolution of thrombi *in vivo*. Thrombolytic agents
35   include tissue plasminogen activator (*se* EP-B 0 093 619),
activase, alteplase, duteplase, silteplase, streptokinase,
anistreplase, urokinase, heparin, warfarin and coumarin.
Additional thrombolytic agents includ saruplase and vampire

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bat plasminogen activator. *See* Harris, *Protein Engineering* 6
(1987) 449-458; PCT/EP 90/00194; US Patent 4,970,159)
(incorporated by reference in their entirety for all
purposes).

5   The combined use of anti-L-selectin agents and
thrombolytic agents is particularly useful for treating
patients with acute myocardial infarction. In conventional
methods, the obstructed coronary artery may be reopened by
administration of thrombolytic agents alone. These agents
10   lyse the clot causing the obstruction, thereby restoring
coronary blood flow. Alternatively, conventional methods
restore blood flow by acute percutaneous transluminal coronary
angioplasty (PTCA), a procedure in which an obstructed and
narrowed segment of the coronary artery is dilated with a
15   balloon. However, restoration of coronary blood flow by
either of these approaches can lead to ischemia-reperfusion
injury.

          In the present methods, ischemia-reperfusion injury
is reduced or prevented by combination of a thrombolytic agent
20   or of PTCA with humanized or human anti-L-selectin antibodies.
Antibodies are usually administered prophylactically before or
at the same time as administration of thrombolytic agents or
initiation of PTCA. Further doses of antibody are then often
administered during and after thrombolytic or angioplastic
25   treatment. The interval between prophylactic administration
of the antibodies and initiation of thrombolytic or
angioplastic treatment is usually 5-30 min, preferably 5-20
min, and most preferably 5-10 min.

          Thrombolytic agents are administered to a patient in
30   an amount sufficient to partially disperse, or prevent the
formation of, thrombi and their complications. An amount
adequate to accomplish this is defined as a "therapeutically
effective dose" or "efficacious dose." Amounts effective for
this use will depend upon the severity of the condition, the
35   general state of the patient, the rout of administration and
combination with other drugs. Often, therapeutically
effective doses of thrombolytic agents and administration
regimens for such agents are thos approved by the FDA, for

independent uses of thrombolytic agents, *e.g.*, 100 mg of
alteplase or 1.5 million IU of streptokinase.

The antibodies of the invention can also be used in
conjunction with chemotherapeutic agents. Typically, the
agents may include non-steroidal anti-inflammatory drugs
(*e.g.*, aspirin) and corticosteroids, but numerous additional
agents (*e.g.*, cyclosporin) well-known to those skilled in the
art of medicine may also be utilized.

The invention also provides methods of reperfusion
therapy in which anti-L-selectin agents are conjugated to
toxin moieties. In these methods, neutrophils expressing L-
selectin are not merely inhibited, but eliminated, by the
toxin moiety. The therapeutic agent component provides a
means for delivering the toxic agent to cells expressing an L-
selectin epitope. The two components can be bonded by
heterobifunctional cross-linkers, *e.g.*, SPDP, carbodiimide,
glutaraldehyde, or the like. The components may also be
linked genetically (*see* Chaudhary et al., *Nature* 339:394
(1989), incorporated by reference in its entirety for all
purposes). Other methods of producing toxin conjugates are
discussed by Thorpe et al. in *Monoclonal Antibodies in
Clinical Medicine* (Academic Press, 1982), pp. 168-190, which
is incorporated by reference in its entirety for all
purposes).

A variety of cytotoxic agents are suitable for use
in immunotoxins. Cytotoxic agents can include radionuclides,
such as Iodine-131 or other isotopes of iodine, Yttrium-90,
Rhenium-188, and Bismuth-212 or other alpha emitters; a number
of chemotherapeutic drugs, such as vindesine, methotrexate,
adriamycin, and cisplatin; and cytotoxic proteins such as
ribosomal inhibiting proteins like pokeweed antiviral protein,
Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A
chain, etc., or an agent active at the cell surface, such as
the phospholipase enzymes (*e.g.*, phospholipase C). (*See,
generally*, commonly assigned U.S.S.N. 07/290,968, "Chimeric
Toxins," Olsnes & Phil, *Pharmac. There.*, 25:355-381 (1982),
and *Monoclonal Antibodies for Cancer Detection and Th rapy*
(eds. Baldwin and Byers, Academic Press, 1985), pp. 159-179,

224-266, all of which are incorporated herein in their
entirety by reference for all purposes.)

The delivery component of the immunotoxin will
include the immunoglobulins discussed *supra*. Intact

5   immunoglobulins or their binding fragments, such as Fab or Fv,
are preferably used. Typically, the antibodies in the
immunotoxins will be of the human IgM or IgG isotype, but
other mammalian constant regions may be utilized as desired.

The therapeutic agents and compositions of the

10 invention are preferably administered before commencement of
reperfusion. Indeed, in situations where the likelihood of
ischemia is predictable (*e.g.*, elective heart surgery), the
therapeutic agents can be advantageously administered before
the blockage to the blood supply occurs. Additional dosages

15 of therapeutic agent may be administered throughout the
reperfusion period and for several hours or days thereafter.

Therapeutic agents and compositions are administered
in amount such as to prevent or at least detectably reduce the
tissue damage and its complications that would otherwise

20 result from reperfusion therapy. An amount adequate to
accomplish this is defined as a "therapeutically effective
dose." Amounts effective for this use will depend upon the
severity of the disease and the general state of the patient's
own immune system, but generally range from about 0.01 - 10

25 mg/kg body weight, preferably of 0.14 - 5 mg/kg. and most
preferably of 0.3 - 3 mg/kg. The antibodies can be given as
an intravenous bolus injection, *e.g.*, over 1 - 5 min.,
repeated injections of smaller doses, or as an intravenous
infusion. The bolus injection is especially useful before

30 commencing reperfusion therapy, or in an emergency. Further
doses of antibodies can be repeated (*e.g.*, every 4 - 6 h)
during and after reperfusion therapy at the same doses as
described above to achieve optimal plasma levels of the
antibody. Because many of the therapeutic agents of the

35 inventi n compris substantially or entirely human
polypeptides, they can be tolerated in high dosages.

The therapeutic agents and pharmaceutical
c mpositi ns th reof of this invention are usually

administered parenterally, *i.e.*, subcutaneously,
intramuscularly or, intravenously. Intravenous administration
is preferred. The agents can also be administered, typically
for local application, by gavage or lavage, intraperitoneal
5   injection, ophthalmic ointment, topical ointment, intracranial
injection (typically into a brain ventricle), intrapericardiac
injection, or intrabursal injection.

                              EXAMPLES
10   Example 1: Cross-Reactivity of hu DREG-200 with Feline PMNs
             Binding of humanized MAb DREG-200 of IgG4 isotype
     (hu DREG-200) to L-selectin on cat neutrophils was determined
     by flow cytometric analysis employing a Becton-Dickinson FACS™
     flow cytometer. Five ml of whole cat blood was collected into
15   vacutainer tubes (containing 50 μl of 15% ethylene diamine
     tetracetic acid EDTA, Becton-Dickinson, Rutherford, NJ) and
     stored at 4°C. 200 μl of the anticoagulated blood was
     incubated with 40 μg/ml of hu DREG-200 or the control MAB
     isotype-matched, humanized ABL-364 (hu ABL-364) at 4°C for 30
20   min. Blood was centrifuged at 500g for 15 min at 4°C
     (Brinkmann Instruments Co.), the supernatant was discarded,
     and the pellet was washed with Dulbecco's PBS (Sigma Chemical
     Corp.) containing 0.2% bovine serum albumin (BSA) (Sigma
     Chemical Corp). The supernatant was removed following
25   centrifugation and F(ab')2 fragments of a goat anti-mouse IgG-
     phycoerythrin conjugate (Tago, Inc., Burlingame, CA) (i.e.,
     1:100 dilution) was added as a secondary antibody for 30 min
     at 4°C. Following an additional wash with 0.2% BSA in PBS,
     the red cells were lysed utilizing a whole blood lysis kit
30   (Coulter Immunology, Hialeah, FL). The remaining neutrophils
     were washed and fixed with 1% paraformaldehyde (Sigma Chemical
     Corp.). The binding of hu DREG-200 to PMNs was determined by
     appropriate gating of the neutrophil population based on cell
     size and granularity of the dot plot. Histograms were
35   generated by cell number versus fluorescenc intensity of at
     least 10,000 cells per sample.
             It was found that 92.5% of the neutrophils stained
     positively with hu DREG-200 compared to 3±2% p sitive staining

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with the control hu ABL-364. Binding of hu DREG-200 to feline
neutrophils resulted in an increase of m an channel
fluorescence of 82±3 compared to 5±2 for hu ABL-364 (p<0.01).
It is concluded that hu DREG-200 binds to L-selectin on cat
5   neutrophils.

In an analogous experiment using the murine
antibody, mouse DREG-200 (from which hu DREG-200 was derived),
it was found that unstimulated cat neutrophils stained 75.0
+/- 9.3% positive compared with 5.1 +\- 0.3% positive staining
10   for a control antibody. See Ma et al, *Circulation* 88:649-57
(1993) (which is incorporated by reference in its entirety for
all purposes).

Example 2:   Inhibition of Adherence by hu DREG-200 *In Vitro*
15   Peripheral blood was collected from the femoral
artery of four cats and anticoagulated with citrate-phosphate-
dextrose solution. PMNs were isolated and labeled as
described above. Cats were subjected to 90 min of ischemia
and 20 min of reperfusion. Hearts were removed and placed in
20   warm, oxygenated K-H buffer. Both the ischemic-reperfused LAD
and non-ischemic LCX coronary arteries were removed and cut in
2 to 3 mm segments. The arteries were then opened, and placed
into a cell culture dish filled with 3 ml of K-H buffer. The
labeled neutrophils (i.e., 1.2 x 10^6/ml) were incubated with
25   different concentrations of hu DREG-200 (i.e., 5, 10, 20 or 40
μg/ml) or the control hu ABL-364 (40 μg/ml) for 10 min at 4°C.
Following the incubation, the PMNs were washed twice with cold
K-H solution. Neutrophils were added into K-H solution of the
cell culture dishes containing the coronary arteries to yield
30   a PMN count of 4 x 10^5/ml. After the incubation period, the
coronary rings were removed and placed onto glass microscope
slides. PMNs were counted using epifluorescence microscopy
(Nikon Corp., Japan). Adherent neutrophils were counted on
five fields from each vessel segment, averaged, and expressed
35   as PMNs/mm^2.

Fig. 2 shows that increased adhesion of unstimulated
PMN adh r nce to coronary artery endothelium following
ischemia/reperfusion was significantly (p<0.01) inhibited by

10 to 40 μg/ml of hu DREG-200 in a concentration dependent
manner. By contrast, hu ABL-364 had no significant eff ct n
PMN adherence to the ischemic-reperfused endothelium. These
results confirm that L-selectin, contributes significantly to
the interaction between PMNs and the coronary endothelium
post-reperfusion, and that this interaction can be blocked by
hu DREG-200.

          In an analogous experiment with the mouse DREG-200
antibody, similar results were obtained. Mouse DREG-200
inhibited PMN adherence to the endothelium in a concentration-
dependent manner with a maximal inhibition of about 70%
occurring at 20 μg/ml of mouse DREG-200. See Ma et al.
(1993), supra.

Example 3: Effect of hu DREG-200 on Myocardial Injury
Following Reperfusion
          The effect of hu DREG-200 on the degree of actual
myocardial salvage of ischemic tissue following reperfusion
was investigated. Adult male cats (2.8-4.2 kg) were
anesthetized with sodium pentobarbital (30 mg/kg, i.v.). An
intratracheal cannula was inserted through a midline incision,
and the cats were placed on intermittent positive-pressure
ventilation (Harvard small animal respirator, Dover, MA). A
polyethylene catheter was inserted into the right external
jugular vein for additional pentobarbital infusion in order to
maintain a surgical plane of anesthesia and for administration
of antibodies. Another polyethylene catheter was inserted
through the left femoral artery and positioned in the
abdominal aorta for the measurement of mean arterial blood
pressure (MABP) via a pressure transducer (Cobe Instruments,
Lakewood, CO). After a midsternal thoracotomy, the anterior
pericardium was incised, and a 3-0 silk suture was placed
around the left anterior descending (LAD) coronary artery 8 to
10 mm from its origin. A high-fidelity catheter tip pressure
transducer (Model MPC 500, with transducer control unit -
Model TCB 500, Millar Instruments Inc., Houston, TX) was
introduced into the left ventricle through the apical dimple.
The cath ter was positioned via observation of th LV pr ssur

and dP/dt wave forms and then secured in place by a silk
suture. Standard lead II of the scalar electrocardiogram
(ECG) was used to determine heart rate (HR) and ST-segment
elevation. ST-segment elevations were determined by analysis
5   of the ECG recording at 50 mm/sec every 20 min. The ECG,
MABP, LVP and dP/dt were continuously monitored on a Hewlett-
Packard 78304 A unit (Hewlett-Packard, Palo Alto, CA) and
recorded on a Gould oscillographic recorder (Gould Inc.,
Cleveland, OH) every 20 min. The pressure-rate index (PRI),
10   an approximation of myocardial oxygen demand, was calculated
as the product of MABP and HR divided by 1000.

          After completing all surgical procedures, the cats
were allowed to stabilize for 30 minutes, at which time
baseline readings of ECG, MABP, LVP and dP/dt were recorded.
15   Myocardial ischemia (MI) was induced by tightening the
initially placed reversible ligature around the LAD so that
the vessel was completely occluded. This was designated as
time point zero. Two mg/kg of hu DREG-200 (IgG4 isotype, as
in previous examples) or a control hu ABL-364 (i.e.,
20   isotypematched humanized control IgG4 MAb) was given
intravenously as a bolus 80 min after coronary occlusion
(i.e., 10 min prior to reperfusion, R). Ten minutes later
(i.e., after a total of 90 min ischemia, I) the LAD ligature
was untied and the ischemic myocardium was reperfused for
25   4.5 h.

          The cats were randomly divided into three major
groups. Six sham MI + R cats received hu DREG-200 (2 mg/kg),
six MI + R cats received the control hu ABL-364 (2 mg/kg), and
six MI + R cats received hu DREG-200 (2 mg/kg). Sham MI + R
30   cats were subjected to the same surgical procedures as MI + R
cats except that the LAD coronary artery was not occluded.

          At the end of the 4.5 h reperfusion period, the
ligature around the LAD was again tightened. 20 ml of 0.5%
Evans blue was rapidly injected into the left ventricle to
35   stain th area of myocardium which was perfused by the pat nt
coronary arteries. The area-at-risk was determined by
negative staining. Immediately following this injecti n, the
heart was rapidly excised and placed in warmed, oxygenated K-H

solution. The left circumflex (LCX) and the LAD coronary
arteries were isolated and removed for subsequent study of
coronary ring vasoactivity and PMN adherence. The right
ventricle, great vessels, and fat tissue were carefully

5   removed, and the left ventricle was sliced parallel to the
atrioventricular groove in 3 mm thick sections. The unstained
portion of the myocardium (i.e., the total area-at-risk or
ischemic) was separated from the Evans-blue-stained portion of
the myocardium (i.e., the area-not-at-risk or non-ischemic).

10   The area-at-risk was sectioned into small cubes and incubated
in 0.1% nitroblue tetrazolium in phosphate solution at pH 7.4
and 37°C for 15 min. The tetrazolium dye forms a blue
formazan complex in the presence of myocardial cells
containing active dehydrogenases and their cofactors. The

15   irreversibly injured or necrotic portion of the myocardium-at-
risk which did not stain was separated from the stained
portion of the myocardium (i.e., the ischemic but non-necrotic
area). The three portions of the myocardium (i.e., non-
ischemic, ischemic non-necrotic, and ischemic necrotic tissue)

20   were subsequently weighed. Results were expressed as necrotic
cardiac tissue area as a percentage of either the area-at-risk
or of total left ventricular mass.

            According to both of these criteria, cardiac tissue
damage was significantly attenuated (p<0.001) in cats treated

25   with hu DREG-200. Similar results have been obtained in an
analogous experiment using the mouse DREG-200 antibody. See
Ma et al. (1993), supra. For hu DREG-200, whereas about 30%
of the jeopardized myocardium developed into necrotic tissue
in the group treated with the control antibody, the amount of

30   necrotic tissue was less than 15% (p<0.01) in the hu DREG-200
treated group, a decrease of 50-60%. See Fig. 3. There was
no significant difference in the wet weights of the areas-at-
risk expressed as a percentage of total left ventricle between
the two ischemic groups (Fig. 3), indicating that a comparable

35   area of myocardial ischemia occurred in both groups.
Therefore, hu DREG-200 significantly protects against
reperfusion injury.

The remarkable preservation of ischemic tissue by hu
DREG-200 is further illustrated from measurements of plasma
creatine kinase activity, a biochemical marker of myocardial
injury. Arterial blood samples (2 ml) were drawn immediately
5   before ligation and hourly thereafter. The blood was
collected in polyethylene tubes containing 200 IU of heparin
sodium. Samples were centrifuged at 2000 x g and 4°C for 20
min and the plasma was decanted for biochemical analysis.
Plasma protein concentration was assayed using the biuret
10 method of Gornall et al. (J. Biol. Chem. 177:751-766 (1949)).
Plasma creatine kinase (CK) activity was measured using the
method of Rosalki (J. Lab. Clin Med. 69:696-705 (1967)) and
expressed as IU/µg protein.

In sham MI/R cats receiving hu DREG-200, the plasma
15 CK activity increased slightly throughout the 6 hour
observation period reaching a final value of 3.8 ± 0.9 IU/µg
protein. In the two ischemic groups, plasma CK activity
increased slightly during the period of myocardial ischemia.
In cats receiving hu ABL-364, CK activity in circulating blood
20 increased markedly within the first 30 min follow reperfusion
and further increased during the remaining four hours of
reperfusion. By contrast, ischemic cats treated with hu DREG-
200 developed significantly lower plasma CK activities
compared with ischemic cats receiving the hu ABL-364 (p<0.05).
25 See Fig. 4. The effect was sustained over the entire
reperfusion period further evidencing the substantial
protection conferred by hu DREG-200 against myocardial
reperfusion injury.

30 Example 4:   Effect of hu DREG-200 on Cardiac Function
Left ventricular pressure (LVP) and the first
derivative of LVP, dP/dt max, an index of myocardial
contractility, were measured by a catheter tip manometer
inserted in th left ventricular cavity. All three groups
35 showed comparable initial values for these cardiac variables.
In the sham MI group ther were no significant changes in
dP/dt max over the entire six hour experimental period.
However, in both MI/R groups, dP/dt max decreased upon

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occlusion of the LAD to about 65%. In cats given hu ABL-364,
contractility did not significantly recover. However, in hu
DREG-200 treated MI-R cats, dP/dt max recovered to control
values three hours following reperfusion. Hence, after 4.5
5   hours of reperfusion, dP/dt max was significantly lower in hu
ABL-364 treated cats than in hu DREG-200 treated cats (p<0.01)
(Fig. 6). These results indicate that hu DREG-200 not only
prevented myocardial necrosis following reperfusion of the
ischemic myocardium, but this myocardial salvage was also
10   translated into an improvement in mechanical performance of
the heart.

Example 5:   Effect of hu DREG-200 on Coronary Endothelial
Dysfunction
15   Since endothelial dysfunction is an early and
critical event in reperfusion injury, endothelial dysfunction
was tested by comparing vasoactivity of isolated coronary
artery rings to the endothelium-dependent vasodilators, ACh
and A-23187, and to the endothelium-independent vasodilator,
20   $NaNO_2$.
             Both LAD and LCX coronary segments were removed and
placed into warmed K-H buffer. Isolated coronary vessels were
cleaned and cut into rings 2 to 3 mm in length. The rings
were then mounted on stainless steel hooks, suspended in 10 ml
25   tissue baths, and connected to FT-03 force displacement
transducers (Grass Instrument Co., Quincy, MA) to record
changes in force on a Grass Model 7 oscillographic recorder.
The baths were filled with 10 ml of K-H buffer and aerated at
37°C with a gas mixture of 95% O_2 and 5% CO_2. Coronary rings
30   were initially stretched to give a preload of 0.5 grams of
force and equilibrated for 60-90 minutes. During this period,
the K-H buffer in the tissue baths was replaced every 15 min.
After equilibration, the rings were then exposed to 100 nM U-
46619 (9,11-epoxymethano-PGH_2, Biomol Research Laboratories,
35   Plymouth Meeting, PA), a thromboxane A_2 mimetic, to generate
ab ut 0.5 g of developed force. Onc a stable c ntraction was
obtained, acetylcholine (ACh), an endothelium-dependent
vasodilator, was added to the bath in cumulative

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concentrations of 0.1, 1, 10, and 100 nM. After the response
stabilized, the rings were washed and allowed to equilibrate
to baseline once again. The procedure was repeated with
another endothelium-dependent vasodilator, A-23187, (1, 10,
5   100 and 1000 nM) and then again with an endothelium-
independent vasodilator, acidified $NaNO_2$ at pH 2, (0.1, 1, 10,
and 100 μM). Saline (i.e., 0.9% NaCl) titrated to pH 2 did
not relax coronary arteries at all. Relaxation was calculated
as % relaxation from the U46619 induced contraction.

10   Fig. 7 summarizes the vasorelaxant responses to ACh,
A-23187, and NaNO2 of ischemic reperfused LAD coronary rings
or non-ischemic LCX obtained from sham MI + I R cats or MI + R
cats receiving either hu ABL-364 or hu DREG-200. Coronary
vascular rings isolated from sham MI + R cats exhibited full
15   relaxation to the endothelium-dependent vasodilator ACh and A-
23187 as well as to the endothelium-independent vasodilator,
$NaNO_2$. Rings from ischemic reperfused cats receiving the
control MAb also showed unchanged relation to $NaNO_2$, but
relaxation to ACh and A-23187 was significantly retarded,
20   indicating endothelial dysfunction. By contrast, relaxation
to ACh and A-23187 in rings obtained from cats treated with hu
DREG-200 was significantly preserved. Control LCX coronary
rings isolated from all three groups relaxed fully to both the
endothelium-dependent vasodilators (i.e., ACh and A23187) and
25   the endothelium-independent vasodilator, $NaNO_2$. Thus, hu
DREG-200 significantly protects against endothelial
dysfunction observed in coronary artery rings subjected to
myocardial ischemia and reperfusion.
            In an analogous experiment, similar results have
30   been obtained using the mouse DREG-200 antibody. *See* Ma et
al. (1993), *supra*.

Example 6: Inhibition of Neutrophil Accumulation in the
Ischemic-Reperfused Area by hu DREG-200

35   The protective activity of hu DREG-200 is further
manifested by its capacity to prevent accumulation of
neutrophils in the ischemic region during reperfusion.
Neutrophil accumulation is thought to be one of the major

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m chanisms responsible for reperfusion injury. Neutrophil
accumulation was assay d from myeloperodixas (MPO) activity,
an enzyme occurring virtually exclusively in neutrophils, in
the non-ischemic, ischemic and necrotic portions of the
5   myocardium.

        MPO activity was determined using the method of
Bradley et al., *J. Invest. Dermatol.* 78:206-209 (1992) as
modified by Mullane et al., *J. Pharmacol. Methods* 14:157-167
(1985). The myocardium was homogenized in 0.5% HTAB (Sigma
10 Chemical Co., St. Louis, MO) and dissolved in 50 mM potassium
phosphate buffer at pH 6.0 using a Polytron (PCU-2)
homogenizer. Homogenates were centrifuged at 12,500 x g, 2°C
for 30 min. The supernatants were than collected and reacted
with 0.167 mg/ml of O-dianisidine dihydrochloride (Sigma
15 Chemical Co., St. Louis, MO) and 0.0005% H_2O_2 in 50 mM
phosphate buffer at pH 6.0. The change in absorbance was
measured spectrophotometrically at 460 nm. One unit of MPO is
defined as that quantity of enzyme hydrolyzing 1 mmol of
peroxide/min at 25°C.

20   Fig. 5 shows that in the non-ischemic myocardium
(i.e., area-not-at-risk), MPO activity was equally low in both
MI groups. However, MI cats receiving only the control
antibody, hu ABL-364, exhibited a marked increase in MPO
activity in the ischemic region. MPO activity increased in
25 the necrotic portion to 0.8 ± 0.2 IU/μg protein. In contrast,
hu DREG-200-treated ischemic cats exhibited a significantly
lower MPO activity in both ischemic non-necrotic myocardial
tissue (0.12 ± 0.10 IU/μg protein, $p<0.05$) and necrotic
myocardial tissue (0.20 ± 0.12 IU/μg protein, $p<0.01$). These
30 results indicate that hu DREG-200 retards neutrophil
accumulation in the ischemic-reperfused myocardium.

        In an analogous experiment, similar results have
been obtained using the mouse DREG-200 antibody. *See* Ma et
al. (1993), *supra.*

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<u>Example 7:   Absence of Side Effects from hu DREG-200 Treatm nt</u>

In cats subjected to sham myocardial ischemia and
reperfusion, intravenous administration of hu DREG-200
(2 mg/kg) had no detectable effect on any of the measured
hemodynamic or electrocardiographic variables. In the two
groups of MI + R cats (receiving hu DREG-200 or control
antibody), there were no significant differences in any of the
variables observed before coronary occlusion. A few minutes
after LAD occlusion, the ST-segment of the ECG became
significantly elevated in both groups. After reperfusion, the
ST-segment decreased to nearly control values, indicating that
effective reperfusion had occurred. There was no significant
difference in peak ST-segment elevation between the two MI + R
groups (0.25 ±0.04 mV vs. 0.20 ± 0.05 mV for the hu DREG-200
and hu ABL-364 groups, respectively), indicating that the
ischemic insult was similar in these two MI + R groups. At
reperfusion, there was a noticeable increase in the incidence
of premature ventricular contractions in all cats. Three cats
in the MI + R group treated with hu ABL-364 and two in the hu
DREG-200 group developed ventricular fibrillation prior to
antibody treatment which was successfully converted to a
normal sinus rhythm by using a DC electronic defibrillator
(Sanborn Co, Waltham, MA). However, there was no obvious
overall difference between the MI + R groups in the number of
PVCs occurring post-reperfusion, indicating that hu DREG-200
did not appear to exert any overt anti-arrhythmic effect. In
both groups of MI + R cats, the PRI decreased significantly
after coronary occlusion and gradually returned to nearly
control values after reperfusion. There was no significant
differences between the two MI + R groups at any of the hourly
PRI readings, suggesting hu DREG-200 did not exert a systemic
hemodynamic effect (i.e., reduced myocardial oxygen demand)
that could account for the cardioprotection.

As a further control to determine whether hu DREG-
200 exerted any leukopenic eff cts, circulating white blood
cells (WBC) were counted ov r the experimental period.
Peripheral WBCs were counted 5 min before coronary occlusion,
5 min before reperfusion, and 30, 150 and 270 min after

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reperfusion. WBC counts did not change significantly over the course of the experiment in either the sham MI group, th hu ABL-364 group or the hu DREG-200 treated group. Furthermore, there were no significant differences in total leukocyte
5   counts among the three groups of cats at any time. All samples checked exhibited 60-65% PMNs of total WBCs. These results show that hu DREG-200 does not produce leukopenia.

The above examples are provided to illustrate the
10   invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill and are encompassed by the claims of the invention. All publications, patents and patent applications cited in the application are hereby incorporated by reference in their
15   entirety for all purposes to the same extent as if each were individually denoted as being so incorporated by reference.

WHAT IS CLAIMED IS:

1.   A method for prevention or treatment of
reperfusion injury in a patient, the method comprising
administering a therapeutically effective dose of an agent
that specifically binds to L-selectin thereby inhibiting
adherence of leukocytes to endothelial cells.

2.   The method of claim 1, wherein the patient is
human.

3.   The method of claim 2, wherein the agent is an
antibody.

4.   The method of claim 3, wherein the antibody is a
monoclonal antibody.

5.   The method of claim 4, wherein the monoclonal
antibody is a humanized antibody.

6.   The method of claim 4, wherein the monoclonal
antibody is a human antibody.

7.   The method of claim 5, wherein the monoclonal
antibody competes with an antibody designated hu DREG-200 for
specific binding to L-selectin.

8.   The method of claim 6, wherein the monoclonal
antibody competes with an antibody designated hu DREG-200 for
specific binding to L-selectin.

9.   The method of claim 7, wherein the humanized
antibody is of IgG4 isotype.

10.   Th method of claim 3, wherein the antibody is
a Fab or (Fab')$_2$ fragment.

11.   The method of claim 5, further comprising the step of administering a therapeutically effective dose of a thrombolytic agent.

12.   The method of claim 6, further comprising the step of administering a therapeutically effective dose of a thrombolytic agent.

13.   The method of claim 5, further comprising the step of administering a therapeutically effective dose of an antibody specific for CD18.

14.   The method of claim 6, further comprising the step of administering a therapeutically effective dose of an antibody specific for CD18.

15.   The method of claim 3, wherein the patient is suffering from myocardial ischemia.

16.   The method of claim 3, wherein the patient is undergoing angioplasty.

17.   The method of claim 3, wherein the patient is suffering from renal, cerebral, splenic or hepatic ischemia.

18.   A method for prevention or treatment of reperfusion injury in a patient, the method comprising administering a therapeutically active dose of a monoclonal antibody that specifically binds to an epitope of L-selectin responsible for adherence of leukocytes to endothelial cells.

19.   The method of claim 18, wherein the monoclonal antibody competes with an antibody designated hu DREG-200 for specific binding to L-selectin.

20.   The method of claim 19, wherein the patient is suffering from myocardial ischemia.

```
A
  1   D  I  V  M  T  Q  S  P  S  S  L  A  M  S  V  G  Q  K  V  T
  1   D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R  V  T

 21   M  T  C  K  S  S  Q  S  L  L  N  S  S  N  Q  K  N  Y  L  A
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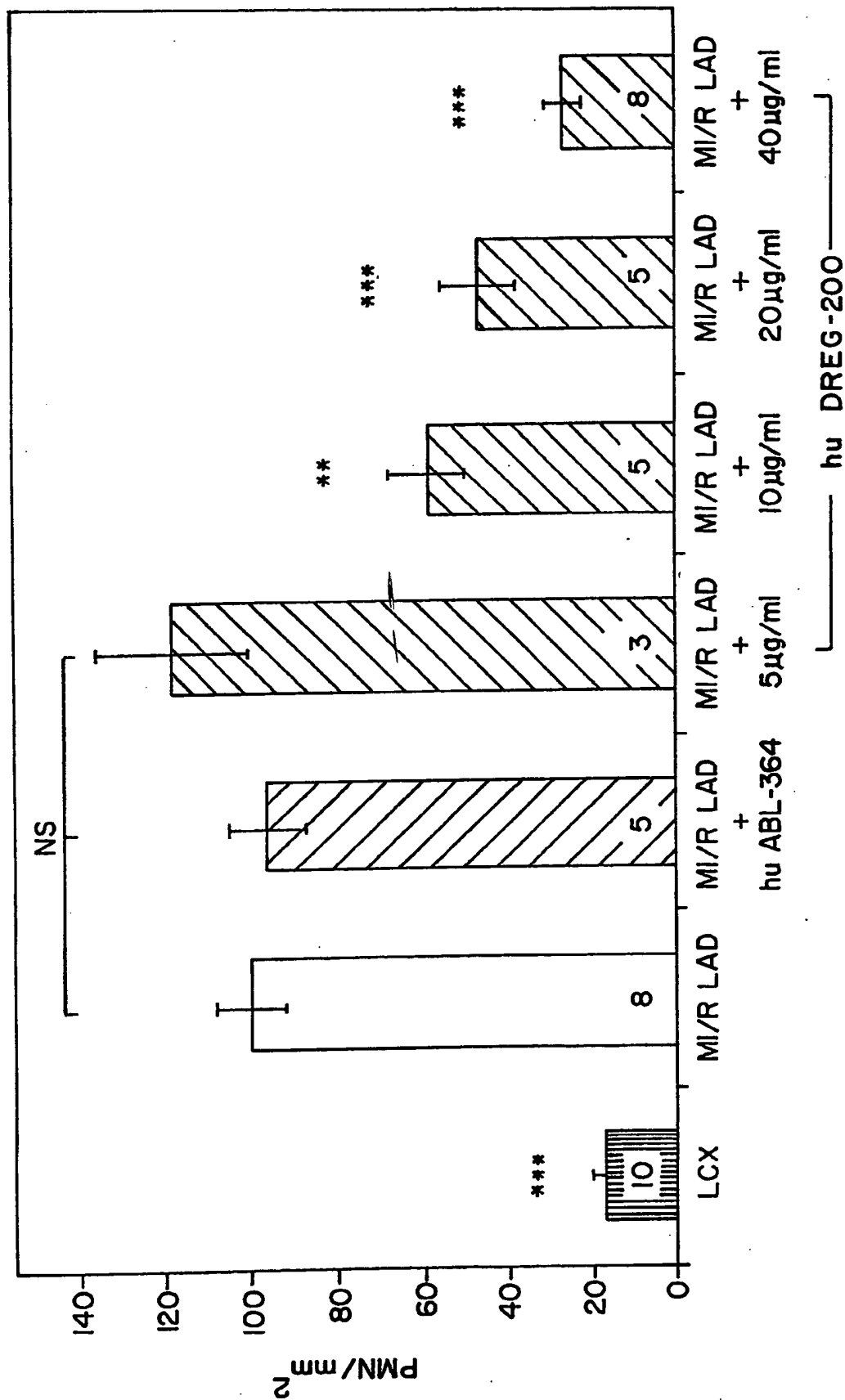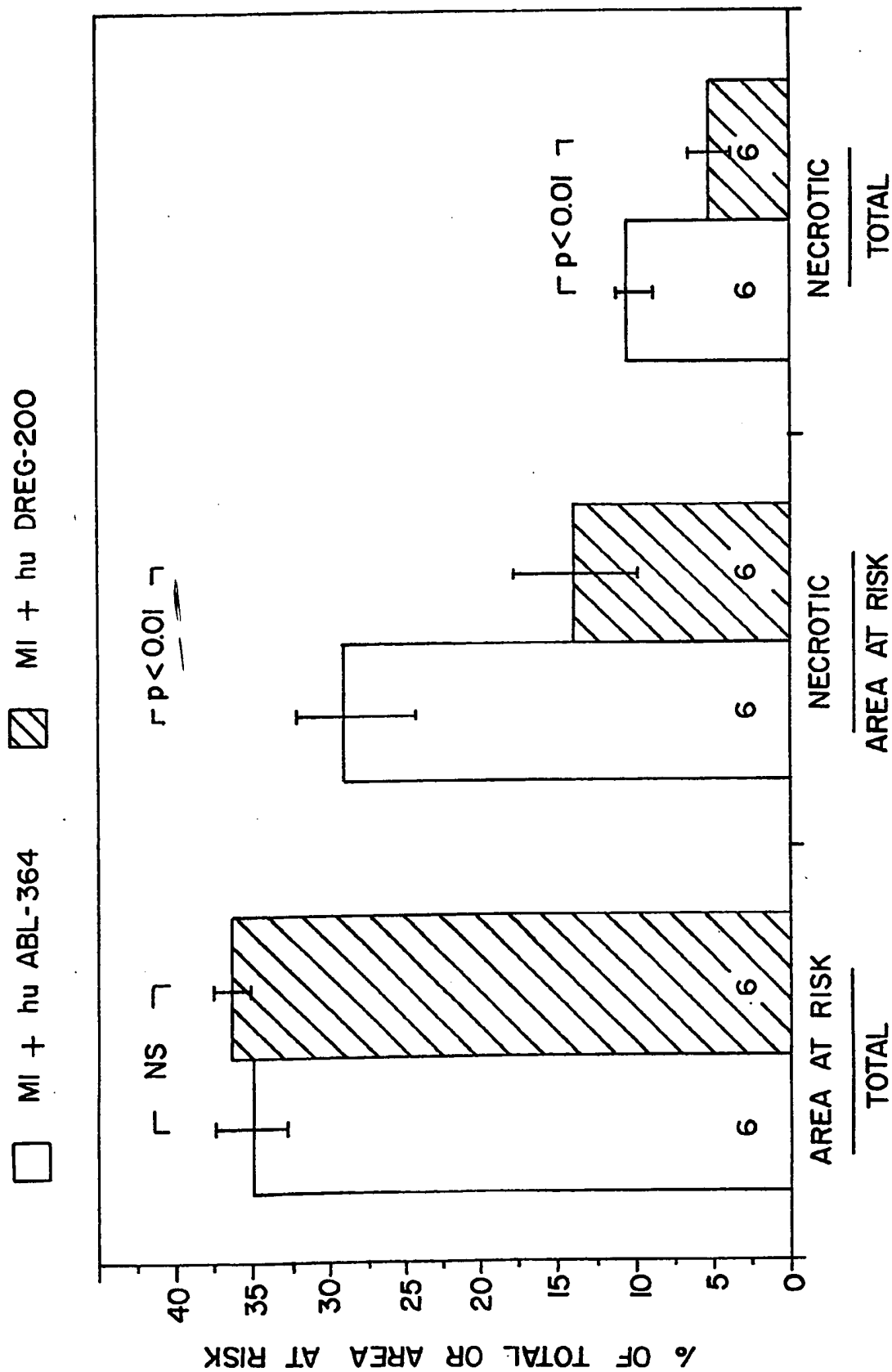
FIG. 1.

FIG. 2.

FIG. 3.
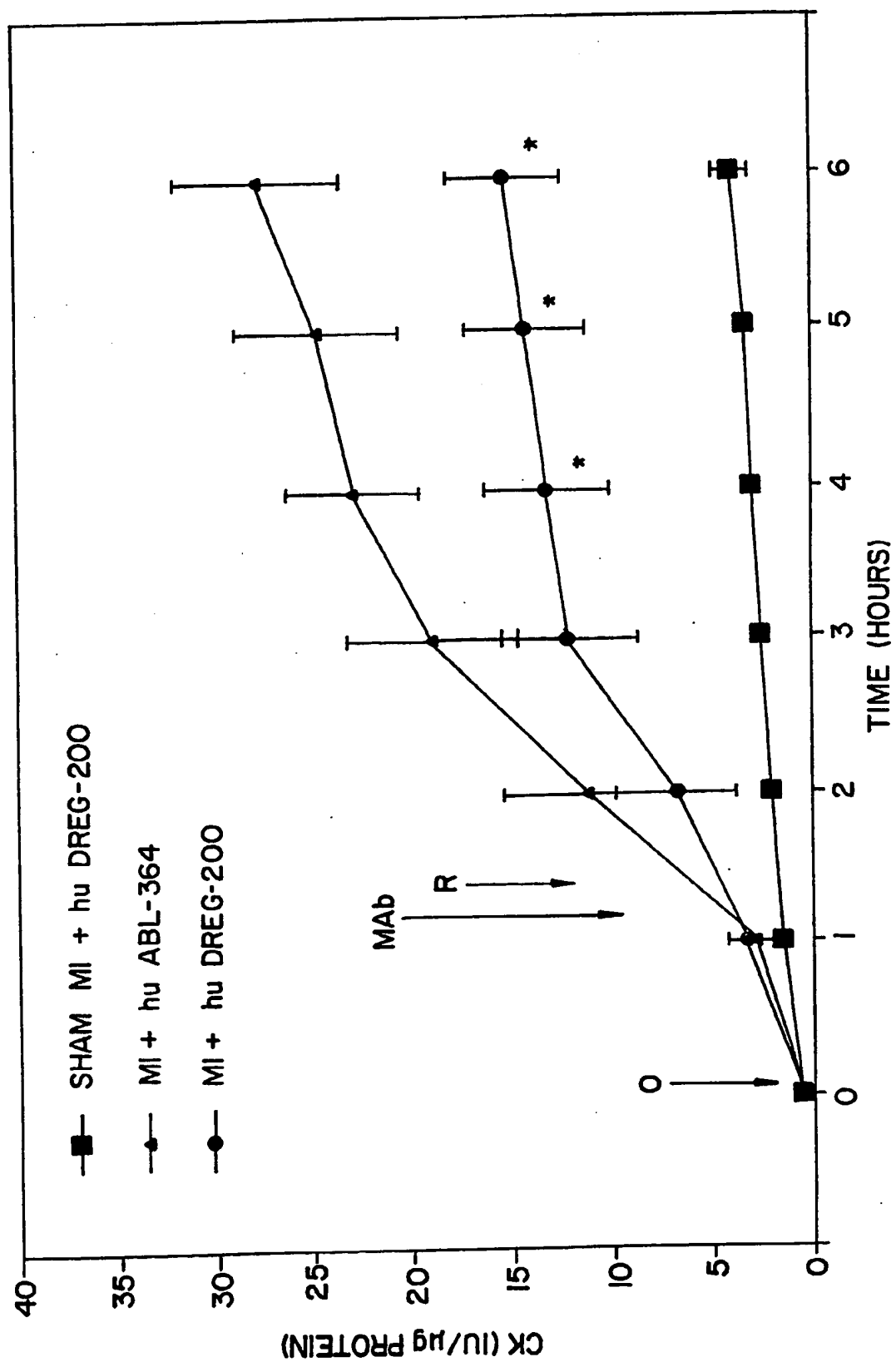
FIG. 4.
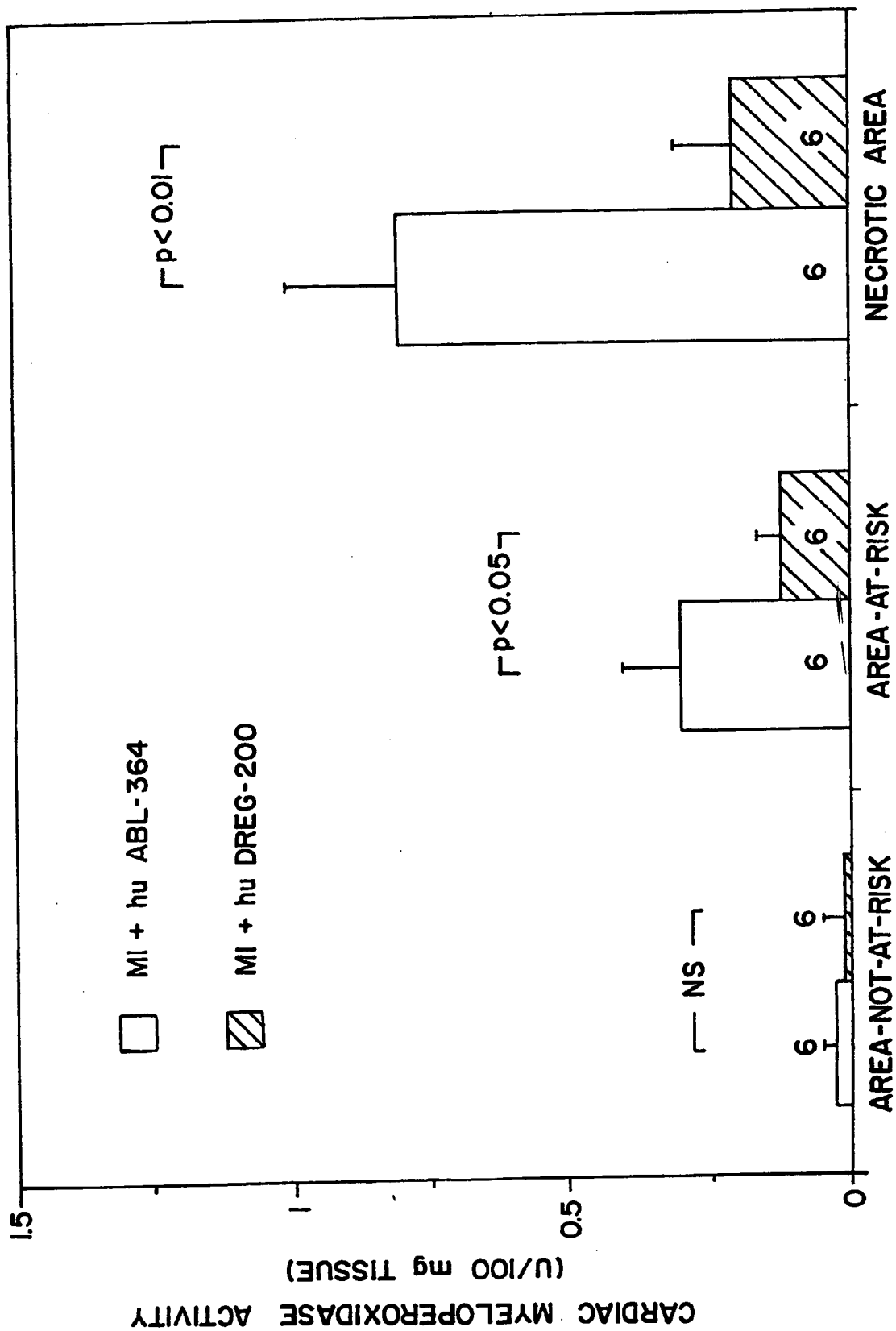
FIG. 5.
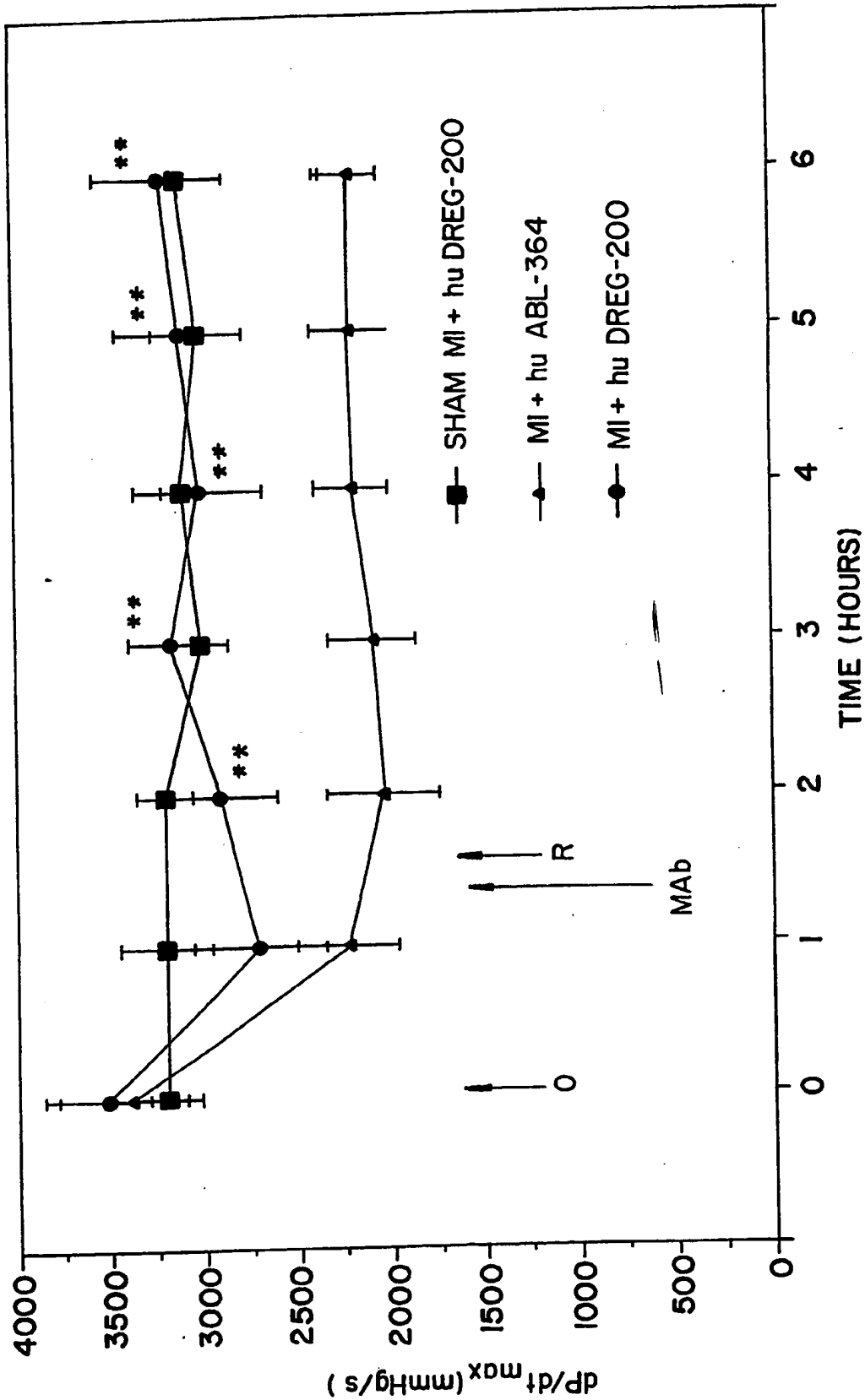
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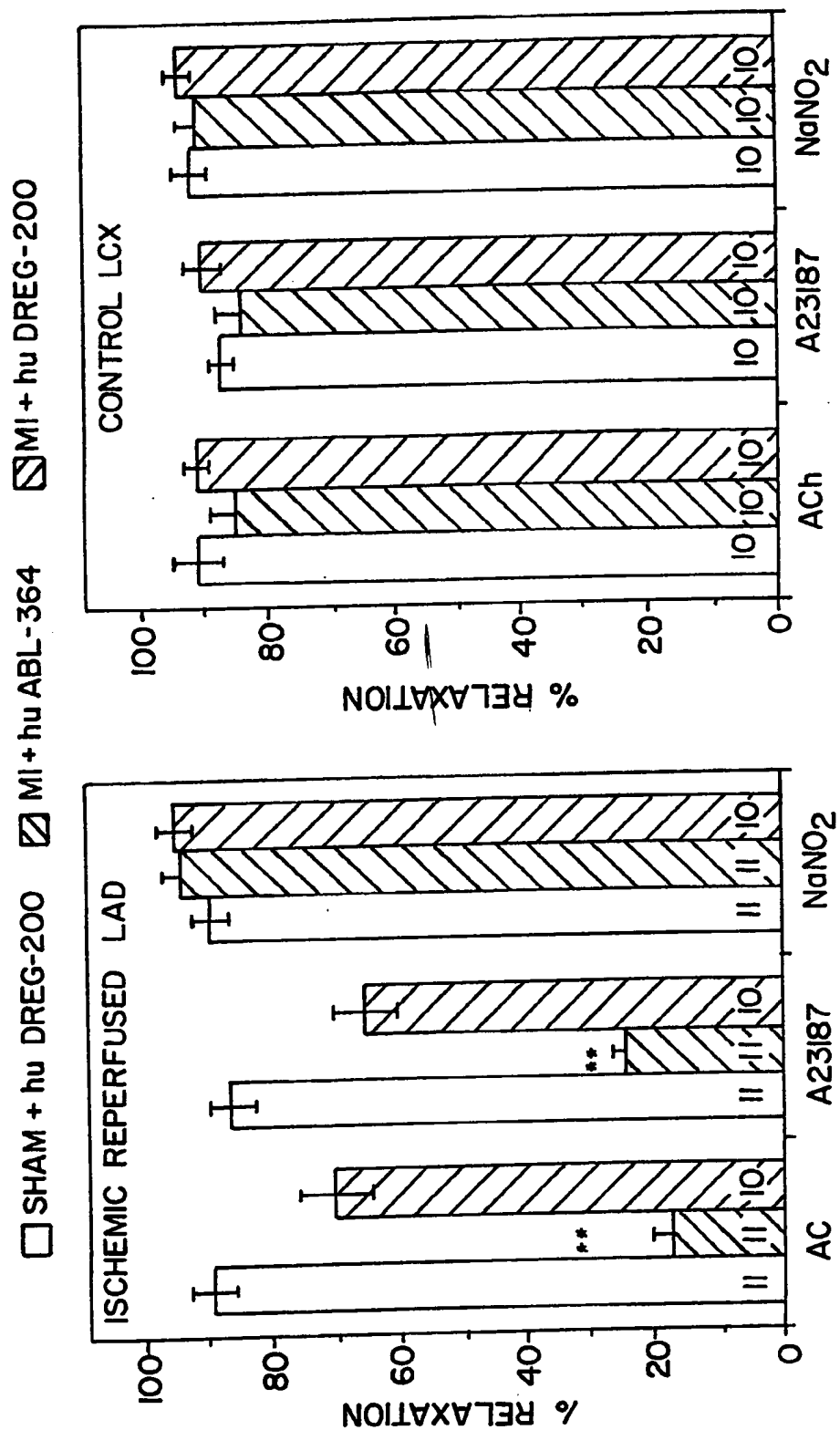
FIG. 7.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; C07K 16/00, 16/18, 16/28
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 133.1, 139.1, 141.1, 144.1, 152.1, 153.1, 154.1; 530/387.3, 388.7, 388.73, 388.75

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, EMBASE, MEDLINE, CHEM ABSTRACTS , WPI
search terms: L-selectin, LAM, DREG-200, CD18

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,797,277 (AFORS) 10 JANUARY 1989, SEE ENTIRE DOCUMENT.	1-20
Y	WO, A, 90/07861 (QUEEN ET AL.) 26 JULY 1990, SEE ENTIRE DOCUMENT.	1-20
Y	SCIENCE, VOLUME 252, ISSUED 21 JUNE 1991, WALDMANN, "MONOCLONAL ANTIBODIES IN DIAGNOSIS AND THERAPY", PAGES 1657-1662, SEE ENTIRE DOCUMENT.	1-20
Y	IMMUNOLOGICAL REVIEWS, VOLUME 114, ISSUED 1990, CARLOS ET AL., "MEMBRANE PROTEINS INVOLVED IN PHAGOCYTE ADHERENCE TO ENDOTHELIUM", PAGES 5-21, SEE ENTIRE DOCUMENT.	1-20

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion f the international search	Date f mailing of the international search report
20 FEBRUARY 1995	**13 MAR 1995**

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLOOD, VOLUME 78, NUMBER 3, ISSUED 01 AUGUST, 1991, KISHIMOTO ET AL., "ANTIBODIES AGAINST HUMAN NEUTROPHIL LECAM-1 (LAM-1/LEU-8/DREG-56 ANTIGEN) AND ENDOTHELIAL CELL ELAM-1 INHIBIT A COMMON CD18-INDEPENDENT ADHESION PATHWAY IN VITRO, PAGE 805-811, SEE ENTIRE DOCUMENT.	1-20
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 88, ISSUED 1991, VON ANDRIAN ET AL., "TWO-STEP MODEL OF LEUKOCYTE-ENDOTHELIAL CELL INTERACTION IN INFLAMMATION: DISTINCT ROLES FOR LECAM-1 AND THE LEUKOCYTE β_2 INTEGRINS *IN VIVO*", PAGES 7538-7542, SEE ENTIRE DOCUMENT.	1-20
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 87, ISSUED MARCH 1990, KISHIMOTO ET AL., "IDENTIFICATION OF A HUMAN PERIPHERAL LYMPH NODE HOMING RECEPTOR: A RAPIDLY DOWN-REGULATED ADHESION MOLECULE", PAGES 2244-2248, SEE ENTIRE DOCUMENT.	1-20

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 133.1, 139.1, 141.1, 144.1, 152.1, 153.1, 154.1; 530/387.3, 388.7, 388.73, 388.75